CHEMISTRY.—*The fungistatic and fungicidal action of certain organic sulphur compounds.*[1] EDWARD L. EVERITT, Georgetown University School of Medicine, and M. X. SULLIVAN, Chemo-Medical Research Institute, Georgetown University.

Both bacteria and molds are in general useful to man, as for example in decomposing plant and animal debris, which, if allowed to accumulate, would sooner or later be to man's detriment. In both fields, certain forms have become parasitic on man or on the fruits of the field on which man depends directly or indirectly for food and clothing. The part that bacteria may play in the production of pathological condition has become common knowledge and has long been under study by the bacteriologist and the practitioner of medicine.

On the other hand, the relation of common molds to health and disease has been given less attention and study since, unlike the bacteria, molds do not in general cause acute and killing diseases, in man at least. However, Kuchenmeister (1857) lists 14 species of fungi that invade the skin, hair, nails, lungs, or mucous membranes. In the list are included *Tricophyton tonsurans*, which invades the hair follicles, and several species of *Aspergillus*, which infect the auditory duct, finger nails, toe nails, etc. Also ringworms caused by fungi of the *Tricophyton* or *Microsporum* families are often met with in man.

In medical practice attention is often called to Moniliae, fungi that invade the mucous membranes, especially of the mouth. Among the diseases of man attributed to this class of fungi are thrush and sprue. There is some suggestion also that fungi, at times at least, may be involved in such allergic conditions as asthma and hay fever. Thus, Prince, Selle, and Morrow (1935) report findings that indicate that molds may play a causative role in some cases of asthma and hay fever, while Brown (1936) considers that hypersensitiveness to fungi must take its place along with sensitization to pollens, animal epidermis, food, and bacteria in the causation of bronchial asthma, eczema, perennial hay fever, and other allergic conditions.

Since molds may destroy the necessary fruits of the field, causing rots of various kinds, and may also invade the skin, the hair, and lungs of man and are incriminated in certain forms of dermatitis, ringworms, actinomycosis of the lungs, loss of hair in some cases, and occasionally in certain allergics, whatever can be found about the metabolic processes and especially about means of preventing them

[1] The data in this paper are taken from the dissertation presented by Edward L. Everitt in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Georgetown University, 1937. Received January 20, 1940.

from getting a foothold on plants, animals, or man would be decidedly worth while.

It is well known that molds and bacteria may be killed or inhibited in their growth by drying, heat, light, pressure, and by various chemicals. Chemical substances that kill are known as fungicides or bactericides, while those that simply inhibit growth are called fungistatic or bacteriostatic. Some of the chemical compounds used in the prevention of the growth of bacteria and molds are silver nitrate, mercuric chloride, iodine, phenol, cresol, benzoic acid, alcohol, and various simple sulphur compounds. Among the sulphur compounds used are carbon disulphide, hydrogen sulphide, sodium thiosulphate, flowers of sulphur, and colloidal sulphur. Lime and sulphur mixtures have been used for years to prevent the growth of fungi on plants and trees.

Our interest in the possibility of inhibiting the growth of molds that invade man was aroused by results obtained in a study of the changes brought about by certain wilt-producing organisms. In a study of acid-base and oxidation-reduction phenomena, it was noted that sodium thiosulphate added to the medium considerably reduced the growth of the molds. This finding suggested a trial of various organic sulphur compounds because elemental sulphur had long been in use in medical practice in ointments for eczema and various skin conditions and parasitic skin infections, and sulphur baths have long been believed to have medicinal value. A direct bearing on the relation of sulphur to fungi is the work of Lynch (1933), who reports the successful use of a sulphur ointment in the treatment of an *Aspergillus* infection in a scalp lesion caused by the bite of the red bug (*Leptus*), and the review of Roark and Busbey (1935), who list a number of organic sulphur compounds of high value as insecticides.

As pointed out by Roark and Busbey, sulphur in various forms and combinations is one of the most valuable and widely used insecticides and fungicides. In the form of elemental sulphur it is applied to fruit trees and ornamental plants both as a dust and in suspension in water for combating red spiders and fungous diseases.

We had on hand a large number of organic sulphur compounds made or secured in a general study of the relation of sulphur and sulphur compounds to health and disease. Some of these we hoped would be of value in medical and agricultural practice.

Accordingly, some 50 sulphur compounds were tested for their fungicidal action in vitro. The molds used were *Fusarium oxysporum* and *F. lycopersicum*, which cause, respectively, wilt of potato plants and tomato plants; *Aspergillus fumigatus*, which invades the ear of man and occasionally the lungs; *Aspergillus niger*, which spoils foodstuffs and like *fumigatus* may invade man; and the *Penicillium* of Fleming, which according to Reid (1935) generates a material toxic to bacteria. The culture media used and the procedure employed for testing the inhibitory action of the various compounds are detailed in the following sections.

The culture medium—The synthetic culture medium used in this investigation was the same as used by Anderson, Everitt, and Adams (1933) in their study of the carbohydrate metabolism of *Fusarium oxysporum*, which causes wilt of potatoes. It was first used by Tochinai (1920) to study the carbohydrate metabolism of *Fusarium lini*. The composition of the medium is as follows:

Ammonium nitrate	1.00 gm
Magnesium sulphate	0.25 gm
Monopotassium phosphate	0.50 gm
Glucose	20.00 gm
Water to make	1,000 cc

In the present work, to 100-cc portions of this culture medium in 250-cc Erlenmeyer flasks various quantities of the organic sulphur compounds were added, and the flasks were plugged with cotton and sterilized at 15 pounds pressure for 20 minutes. Control flasks containing the medium without addition of the sulphur compounds were sterilized in a similar manner.

Inoculation of the medium.—The stock medium for the development of the molds was Sabouraud's dextrose agar described in Difco Manual, ed. 5, 1935. Spores collected from the agar slants were suspended in sterile distilled water, and 1 cc of the spore suspension was added to each culture flask by means of a sterile pipette. The sulphur compounds tested are listed in Table 1.

Compounds (1)–(13) were obtained from Dr. H. L. Haller, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture. The remaining compounds were at hand at Georgetown University.

Inasmuch as most of these compounds were ineffective, that is, did not inhibit the growth of the molds, the formulas are not given here. Those compounds that were effective will be discussed in detail and their formulas will be given later.

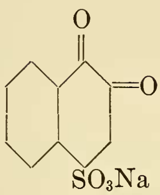
With *Fusarium oxysporum* and *F. lycopersicum* used as test fungi, only a few of the compounds listed in Table 1 inhibited or stopped growth. The inhibitors were Nos. (10), (11), (33), (34), (36), and (47). These compounds inhibited growth for a period varying from 4–15 days, after which time the organism slowly developed. These compounds could be utilized as fungistatic material and might have application in medical practice, in external application. One compound, (24), the disulphide of ortho-thioaminophenol, absolutely prevented growth of *Fusarium oxysporum* but allowed a slight and delayed growth of *Fusarium lycopersicum* with increase in growth after the nineteenth day. Colloidal sulphur, (33) (sulphur diasporal), was labeled as containing 10 mg of S in 2 cc. One cc was added to 100 cc of the culture medium. Colloidal sulphur (sulisocol) was labeled as 2 cc containing 20 mg of sulphur. One-half of 1 cc was added to 100 cc of culture media. Both types of sulphur in a colloidal complex markedly inhibited the growth of the molds, even at concentrations calculated to be 50 mg per liter. Since a question arose

TABLE 1.—SULPHUR COMPOUNDS STUDIED FOR FUNGICIDAL AND BACTERICIDAL PROPERTIES.

(1) Phenothiazine-6-carboxylic acid chloride	(26) Thiazolidine carboxylic acid
(2) 6-Acetyl-phenothiazine	(27) Formyl-dl-cystine
(3) Phenothioxine	(28) S-carboxymethyl-cysteine
(4) Phenothiazine	(29) Cystineamine hydrochloride
(5) Tetrathiopentone	(30) Thiazolidine hydrochloride
(6) Cuprous methylxanthate	(31) Thiobarbituric acid
(7) Cuprous isoamylxanthate	(32) Benzyl disulphide
(8) Chlorbenzoketothiazine	(33) Colloidal sulphur (diasporal) 2 cc = 10 mg
(9) 4-Chloro-2-nitrophenyl thioglycolic acid	(34) Colloidal sulphur (hyposols or sulisocol) 2 cc = 20 mg
(10) Phenyl thioarsenite	(35) Sulphanilic acid
(11) 4-Chloro-2-nitrophenyl sulphur amine	(36) 1,2 Naphthoquinone-4-sodium sulphonate
(12) 4-Chloro-2-nitrophenyl sulphur bromide	(37) 1,2 Naphthoquinone-4-6-sodium disulphonate
(13) Bis(2-nitrophenyl) disulphide	(38) Sodium alizarine sulphonate
(14) Thioacetamide	(39) Tropaolin 000
(15) Mercaptobenzothiazole	(40) Congo red
(16) Phenylbenzothiazole	(41) Bromcresol green
(17) Sodium diethyldithiocarbamate	(42) Methyl orange
(18) Diethyl thiourea	(43) Cresol red
(19) Thiourea	(44) Bromphenol blue
(20) Disulphide of thiotyrosine	(45) Strychnine sulphate
(21) Dithiosalicylic acid	(46) Thymol blue
(22) Phenylthioglycolic-ortho-carboxylic acid	(47) Prontylin (Winthrop)
(23) Benzidine sulphonate	(48) Trional
(24) Disulphide of ortho-thioaminophenol	(49) Sulphonal
(25) Cysteic acid	(50) Cystine
	(51) Sulpharsphenamine

as to whether the inhibiting action was due to the sulphur as such or to the changes in the reaction of the medium, the study of the inorganic sulphur in the protective colloid solution was put aside for later development. Two compounds, mercaptobenzothiazole and phenylbenzothiazole, allowed no growth whatsoever of the two molds mentioned above when present in the culture media at the concentration of 5–10 mg in 100 cc of solution. Because a number of the organic sulphur compounds were found effective in inhibiting or utterly preventing the growth of the two molds, the experiment was extended to other molds as given in Table 2.

An interesting effect of chemical constitution on the growth of the molds was exhibited by compounds (36) and (37), namely, 1, 2 naphthoquinone-4-sodium sulphonate and 1, 2 naphthoquinone-4-6-sodium disulphonate, with the formulae—



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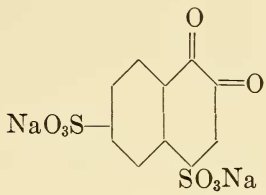


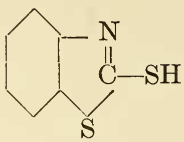
The 1, 2 naphthoquinone-4-sodium sulphonate had marked power of inhibiting the growth of the molds, while the 1, 2 naphthoquinone-4-6-sodium disulphonate was an excellent stimulator of growth. These findings are in harmony with the early work of Ehrlich and Herter (1904), who found the sodium salt of the 1, 2 naphthoquinone monosulphonic acid to be toxic. Since these compounds are relatively expensive and since the work of Ehrlich and Herter, especially that of Herter (1905), indicated that the 1, 2 naphthoquinone-4-sodium sulphonate was toxic to animals, no further attention was given to the compounds as fungistatic or fungicidal substances for use in medical or agricultural practice.

Of the compounds studied in relation to the growth of the molds in vitro,

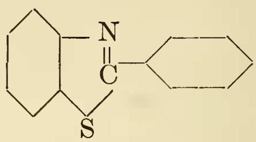
TABLE 2.—STUDY OF THE MOST PROMISING SULPHUR COMPOUNDS HAVING FUNGISTATIC AND FUNGICIDAL PROPERTIES WHEN ADDED TO 100-CC CULTURE MEDIUM.

Compound No.	Amount used	F. oxysporum	F. lycopersicum	A. niger	A. fumigatus	Penicillium of Fleming
	mg					
10	12.5	First growth in 15 days, slow thereafter	No growth in 25 days	No growth in 11 days, slight in 25 days	No growth in 15 days, slight in 25 days	Very few spores in 15 days, slow thereafter
11	12.5	First growth in 15 days	First growth in 15 days	No growth in 11 days, slight in 15 days	No growth in 25 days	A few spores in 15 days, slight thereafter
15	3.0	Growth in 8 days, becoming heavy	Slight growth in 8 days, becoming heavy	Good growth in 8 days, very few spores	No growth in 25 days	Very slight growth in 25 days
15	5.0	No growth at any time	No growth at any time	Slight growth in 8 days, no spores	No growth in 25 days	No growth in 15 days, a few spores in 25 days
15	10.0	No growth	No growth	No growth	No growth	No growth
16	3.0	Slight growth in 8 days, becoming heavy	Slight growth in 8 days, becoming heavy	Heavy growth in 8 days, with spores	Slight growth in 11 days, slowly increasing	Slight growth in 8 days
16	5.0	No growth	No growth	Slight growth in 8 days	No growth in 25 days	Slight growth in 8 days
16	10.0	No growth	No growth	No growth in 25 days	No growth	No growth in 25 days
36	12.5	No growth in 11 days, some spores in 15 days, slowly increasing	No growth in 25 days	Good growth in 8 days	No growth in 25 days	No growth in 15 days, slight growth and a few spores in 25 days
47	10.0	Slight growth in 4 days	Slight growth in 8 days			
47	25.0	No growth in 4 days, slight growth in 12 days	Slight growth in 7 days	Slight growth in 7 days	Slight growth in 7 days	

number (15), mercaptobenzothiazole, and number (16), phenylbenzothiazole, were the most effective. Their chemical constitution, respectively, is—



and



Of these two, mercaptobenzothiazole was on hand in plentiful supply, so some attention was paid to it from chemical and clinical viewpoints and to its possible toxicity toward animals. As may be seen from its formula it is an organic sulphur compound containing nitrogen and an (SH) group. This compound, which is cheap and readily available, was first made by Hofmann (1887) and has been used in the rubber industry as an accelerator of vulcanization for a number of years. Its use for such purposes seems to have been first suggested by Bedford and Sebrell (1921). It is soluble in alcohol, chloroform, and benzol but is not very soluble in water. It is sufficiently soluble in water, however, to be used as a germicide. It is more soluble as a sodium salt and is rather soluble in sodium bicarbonate.

As judged by lack of growth for 50–60 days in the case of *F. oxysporum*, *F. lycopersicum*, and *A. fumigatus*, the compound has marked fungicidal power. In the case of *A. niger* and *Penicillium* of Fleming, mercaptobenzothiazole showed strong fungistatic activity, since no growth occurred until after a period of 25 days. Without prejudice as to whether a compound can be found that is fungistatic or fungicidal toward molds in general, it can be said that with the molds studied by us mercaptobenzothiazole had marked fungicidal or fungistatic activity.

Roark and Busbey (1935) state that mercaptobenzothiazole in concentration of 0.01 to 0.10 percent was effective in controlling a fungus living on wood and that it has been used in controlling aphids and mosquito larvae. Davis (1930) reported that this compound had little if any toxicity. He injected an aqueous solution of it into guinea pigs and a total injection of 14.5 mg in 20 days did not produce any injurious effects on the animals. Medical examination of the men working with mercaptobenzothiazole over a period of years in the Goodyear Tire & Rubber Co. did not show any toxic conditions or dermatoses. In a recent personal communication, Dr. Davis (1939) reiterates the conclusion that the compound has shown no toxic action on men working with it in the vulcanization of rubber.

We have given 20–100 mg of the mercaptobenzothiazole by mouth to guinea pigs weighing 400 grams with no gross evidence of toxicity and have injected 20 mg in aqueous suspension intraperitoneally into a 200-gram guinea pig with no effect on his activity, appetite, or general well-being. Dr. William B. Wardrop, of Washington, D. C., found the mercaptobenzothiazole practically as effective toward "athlete's foot" as salicylic acid. The various sulphur compounds were also tested for their bactericidal and bacteriostatic properties. The findings will be detailed in a subsequent paper.

SUMMARY

About 50 organic sulphur compounds were tested for their fungistatic and fungicidal action.

The molds used were the wilt-producing *Fusarium oxysporum* and *F. lycopersicum*, the pathogenic *Aspergillus fumigatus*, the common *Aspergillus niger*, and the *Penicillium* of Fleming.

Fungistatic activity was manifested by phenylthioarsenite, 4-chloro-2-nitrophenyl sulphur amine, 1, 2 naphthoquinone-4-sodium sulphonate, and prontylin or sulphanilamide.

Fungicidal activity was manifested by mercaptobenzothiazole and phenylbenzothiazole.

The most effective compound was mercaptobenzothiazole, which inhibited the growth of the molds in concentration of 50 to 100 parts per million. This compound is cheap and readily available.

The investigation deals only with in vitro tests, and no conclusion can be drawn as yet as to the therapeutic application of the various compounds. Preliminary toxicity tests on guinea pigs with both oral and intraperitoneal application indicate that mercaptobenzothiazole, the most effective fungicidal compound, has little if any toxicity.

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